EXPERT REVIEWS

Using monoclonal antibodies to stimulate antitumor cellular immunity

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Monoclonal antibodies (mAbs) have an established role in current cancer therapy with seven approved for the treatment of a wide variety of tumors. The approved mAbs directly target tumor cells; however, it is becoming increasingly clear that as well as their direct effects, these mAbs can present antigens to the immune system. This stimulates long-lasting T-cell immunity, which may correlate with long-term survival. A more direct approach is to use mAbs to target antigens directly to antigen-presenting cells. One approach, ImmunoBody®, which has just entered the clinic, stimulates antitumor immunity using mAbs genetically engineered to express tumor-specific T-cell epitopes. T cells not only respond via their T-cell receptors recognizing T-cell epitopes presented on MHC but are also influenced by stimulation of a wide variety of costimulatory molecules. mAbs targeting these molecules can also influence antitumor immunity. The main protagonist in this class of mAbs is ipilimumab, which has recently been shown to improve survival at 2 years in 23% of advanced melanoma patients. Combinations of mAbs targeting tumor antigens to activated antigen-presenting cells and mAbs targeting costimulatory receptors may provide effective therapy for a broad range of tumors.

KEYWORDS: antitumor immunity • cancer vaccines • costimulatory molecules • monoclonal antibodies • T cells

One of the recent goals of monoclonal antibody (mAb) therapy is to stimulate cellular immunity. Induction of cellular immunity begins with uptake of antigens by antigen-presenting cells (APCs) such as dendritic cells (DCs). These cells process antigen and present them as peptides bound to MHC molecules. The peptides can be presented on MHC class I or class II molecules. Typically, exogenous antigens are presented as peptides on class II antigens to the TCR of $CD4^+$ T-helper cells. These cells, when activated, secrete a variety of cytokines to amplify the immune response. Endogenous antigens are processed and presented on MHC class I molecules to the TCR of $CD8^+$ or cytotoxic T cells (CTL). Once activated these cells patrol the body and kill any cell expressing the cognate MHC–peptide complex. More recently, it has been shown that exogenous antigens can also be internalized and can cross-present antigens to the MHC class I pathway stimulating CTL responses. One mechanism of cross presentation is antibody-dependent cellular phagocytosis (ADCP; see later section). Both $CD4^+$ and $CD8^+$ T cells not only respond to TCR stimulation but are also influenced by a wide variety of costimulatory molecules that either amplify or repress TCR signaling. mAbs that either block or stimulate these costimulatory molecules can have a profound effect on immune responses.

Tumors express a range of stress-related molecules that alert the immune system to the danger. This process is termed 'immunosurveillance'. In a recent addition to this theory it has become clear that the transformed cells can acquire further mutations that make them resistant to the immune response. There is then a period of immune equilibrium where the tumor mutates and the immune system adapts to continue to control tumor growth. Ultimately, if the immune system is sculpting the tumor phenotype, a process termed 'immune editing', the tumor may become resistant to immune attack. One consequence of immune editing is alteration of the tumor microenvironments, which become increasingly immunosuppressive. Thus, approaches which relieve this immunouppression, such as mAbs that block negative costimulation of T cells or agonist mAbs which reduce the threshold for T-cell activation, can allow the immune system to resume control or even eliminate the tumor. Alternatively, as the immune response

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usually focuses on a small number of immunodominant antigens, one way of avoiding T-cell attack is to down regulate or mutate these selective antigens. In these situations it will be necessary to re-educate the immune system to recognize a subdominant antigen. This can be done by vaccination. mAbs can be used to target subdominant epitopes/antigens to APCs stimulating potent new antitumor T-cell responses. As the tumor microenvironment may still be immunosuppressive it may be advantageous to combine vaccine approaches with immune regulatory mAbs.

Approved mAbs

A number of differen unconjugated mAbs have been approved for the treatment of cancer and they all have multiple mechanisms of action. Rituximab, a chimeric anti-human CD20 mAb was approved for the treatment of non-Hodgkin's lymphoma (NHL) [1]. Its *in vivo* mechanism of action includes antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), growth inhibition, Fas-mediated apoptosis and chemo- and radio-sensitization of tumor cells [2,3]. Trastuzumab binds to HER2 and is approved for the treatment of HER2-positive breast cancer. Its exact mechanisms of action are not completely understood but include promoting apoptosis and cell cycle arrest, inducing expression of anti-angiogenic factors, suppressing pro-angiogenic factors and mediating ADCC [4–7]. Alemtuzumab is an anti-CD52 mAb approved for the treatment of drug-resistant chronic lymphocytic leukemia . This mAb acts *in vitro* by CDC and ADCC and also induces apoptosis, but its *in vivo* mechanism of action is unclear [8–10]. Cetuximab is directed against HER1 and is approved for the treatment of irinotecan-failed colorectal cancer patients. Cetuximab inhibits proliferation by inhibiting MAPK/PI3K/Akt signaling, upregulating p27, inducing apoptosis, suppressing the production of VEGF and mediating ADCC and CDC [11–13]. Bevacizumab neutralizes VEGF-A and is approved in combination with 5-fluorouracil as first line treatment for metastatic colorectal cancer [14–16]. Finally, panitumumab is also directed against HER1, it has a similar mechanism of action to cetuximab but owing to its subclass (IgG2) is unable to mediate ADCC or CDC and is approved for the treatment of metastatic colorectal cancer [17,18]. Mechanistic studies in FcR$\gamma^{-/-}$ mice confirm the importance of ADCC and CDC for trastuzumab, rituximab and cetuximab [19]. However, there is still partial protection in the FcR$\gamma^{-/-}$ mice or using $F(ab)_2$, suggesting other mechanisms have a role and that they synergize with ADCC and CDC [19,20].

Antibody-dependent cellular phagocytosis

Passively administered mAbs can not only induce direct tumor killing but can also stimulate T-cell responses [21]. Fcγ receptor (FcγR)-mediated phagocytosis can lead to antigen processing and presentation of peptides on their surface MHC for stimulation of T-cell responses. This can be either by direct binding of their Fc domain with FcγR expressed by APCs [22,23] or by deposition of the complement component iC3b, a powerful opsonin, which is recognized by the CR3 receptor expressed by APCs. iC3b/CR3 engagement results in enhancement of FcγR-mediated effector functions. Interestingly, this process is also enhanced by the release of the anaphylatoxins C3a and C5a, which are not only chemotactic for effector cells but selectively increase expression of activating FcγRs relative to the inhibitory FcγRs on APCs [24]

Typically, exogenous antigens are presented on class II MHC and internal antigens on class I MHC. Thus FcγR-mediated phagocytosis would normally lead to presentation on class II MHC leading to activation of $CD4^+$ T-helper cells only; however, recent studies have shown that engagement of FcγRs allows activation of potent tumor killing CTLs, a process referred to as cross-priming. Small immune complexes of antigen and human IgG1 or mouse IgG2a would be internalized via the high affinity Fc receptor, CD64, which has been shown to be very efficient at cross-priming [25]. In contrast, large immune complexes of antigen and human IgG2, human IgG3, mouse IgG2a/c and b, will be internalized via FcγRII or FcγRIV receptors. These are very efficient at stimulating helper responses but are less efficient at cross-priming. Human IgG4 mAbs fail to engage with FcγR receptors and cannot mediate ADCP. There is also the problem of the inhibitory receptors, which bind with equal affinity to both the IgG subclasses and the activating receptors. The inhibitory receptors are preferentially expressed by resting APCs and are down-regulated in favor of the activating receptors by proinflammatory cytokines [26,27]. The advantage of stimulating T-cell responses is that it can, theoretically, lead to long-lasting adaptive antitumor immunity and long-term remission. Indeed, it is such effects that are sometimes invoked to explain the long-term responses observed in lymphoma patients after therapy with the anti-CD20 antibody ritixumab [28,29]. In a small study of ten patients, Her-2/neu-specific $CD4^+$ response could be detected in 60% of patients receiving trastuzumab [30]. More recently, Horlock *et al.* demonstrated that trastuzumab can significantly reduce the regulatory T cell (Treg):Th17 ratio in metastatic breast cancer patients [31]. Intriguingly, the addition of chemotherapeutic drugs to anti-HER2/neu mAbs, although capable of enhancing the reduction of tumor burden, could abrogate antibody-initiated immunity leading to decreased resistance to rechallenge or earlier relapse. Increased influx of both innate and adaptive immune cells into the tumor microenvironment by selected immunotherapy further enhanced subsequent antibody-induced immunity, leading to increased tumor eradication and resistance to rechallenge [32].

Targeting APCs *in vivo*

Antigen-presenting cells are required to stimulate T-cell responses, and hence vaccines targeting these cells should be more effective. Targeting mature DC has been tried both *ex vivo* and *in vitro*. *Ex vivo* DC have been expanded from cancer patients and pulsed with a variety of antigens including peptides, proteins or tumor lysates [33]. Disappointingly, a recent Phase III clinical trial in melanoma failed to show a significant survival advantage for patients immunized with peptide pulsed DC [34]. Clinical trials employing peripheral blood mononuclear cells (PBMC) pulsed with vaccine for the treatment of prostate cancer have had more promising results. A recent placebo controlled Phase III trial in patients with metastatic asymptomatic hormone refractory

prostate cancer reported on the benefits of immunization with autologous PBMC loaded with prostatic acid phosphatase (PAP) antigen in a fusion protein with GM-CSF. Although no improvement in time to disease progression was observed, immunization with the PAP loaded PBMC vaccine demonstrated a statistically significant 4.5 month improvement in overall survival. This was associated with an eightfold improvement in the induction of antigen-specific T-cell responses following immunization with the PAP-loaded PBMC vaccine compared with the placebo control [35]. This vaccine, known as Provenge®, has now been approved by the US FDA.

The ability to target vaccine antigens to DC *in vivo* offers an attractive alternative to the use of autologous DC vaccines, which are patient specific, expensive and difficult to manufacture. Several groups have attempted to achieve this through the use of mAbs to target vaccines to specific receptors on the surface of DCs that facilitate antigen processing and presentation.

Targeting Fcγ receptors on APCs

Several groups have demonstrated enhanced immune responses through targeting of a range of vaccine modalities to the Fc receptors for IgG. It remains unclear how different Ig isotypes interact with Fc receptors. There appear to be high effector function IgGs (human IgG1 and IgG3; murine IgG2a/c and IgG2b) and two apparently low effector functions of IgGs (Human IgG2 and IgG4; murine IgG1 and IgG3) but the balance between activating and inhibitory Fc receptors and their expression on different APCs complicates this picture [21]. DC play a pivotal role in stimulating naive T cells and express FcγRI (CD64, activating), FcγRIIa (CD32a, activating) in humans and FcγRIV (activating) in mice, FcγRIIb (CD32b, inhbitory) and FcγRIIIa (CD16a, activating). The ultimate outcome of FcγR-mediated phagocytosis and cross-presentation appears to depend on the relative engagement of activating versus inhibitory FcγRs. In human *in vitro* studies, blockade of FcγRIIb promotes DC maturation, T-cell activation, and production of IL-12 without the addition of inflammatory cytokines [26,27,36]. IFN-γ modulation of FcγR expression in favor of FcγRIIa also promotes IgG-induced maturation. In mice, blocking FcγRIIb resulted in enhanced tumor immunity using immune complex as the antigen [37].

Immune complexes

Numerous studies in mice have shown a role for Fc targeting in stimulating T-cell responses but none of these approaches have reached the clinic. Akiyama *et al.* showed that immunization with APC pulsed with IgG complexed apoptotic tumor cells enhanced the *in vivo* generation of tumor-specific $CD8^+$ cells and tumor rejection, as compared with APC pulsed with apoptotic tumor cells alone [38]. Rafiq *et al.* showed that tumor immunity specific for ovalbumin (OVA)-expressing tumors could be provided by immunization with OVA-immune complex-pulsed APCs. In contrast, APC deficient in FcγR signaling could not respond to these immune complexes, suggesting that the Fc might also provide FcγR-mediated maturation signals to APC in order to promote immunity rather than tolerance [39]. FcγR targeting has also been reported to improve the efficiency of DNA immunization. Enhanced $CD8^+$ T cells, Th1 and antibody responses were observed *in vivo* following immunization with a DNA construct incorporating an IgG Fc fragment fused to a model hepatitis B antigen compared with a construct encoding the hepatitis B antigen alone [40]. More recently, enhanced immune responses were observed following DNA immunization with a construct encoding Fc that resulted in delayed tumor growth and prolonged survival in a murine model of prostate cancer [41]. Reports in the literature have previously demonstrated that vaccine-induced T-cell responses can be enhanced by mAbs [42,43]. A recent elegant study by Saenger *et al.* demonstrates that antitumor immunity is dramatically enhanced by combination of DNA vaccination and treatment with an anti-TRP-1 antibody [44]. They demonstrate that DNA vaccination elicits $CD8^+$ T-cell responses but these are insufficient to induce an effective antitumor effect. They propose that the immune enhancement observed by combining the DNA vaccine with antibody treatment is Fc receptor-dependent and the adjuvant potency of their antibody could be explained by the activation of Fc receptors and subsequent cross-presentation of tumor antigen.

Anti-idiotypic mAbs

Although antigen/antibody complexes can target APCs to allow stimulation of a cellular immune response, such complexes have proved to be inefficient cancer vaccines due to their difficulty to manufacture and inherent instability. Anti-idiotypic mAbs that mimic specific antigens have been shown to stimulate antibody responses particularly against ganglioside antigens but clinic trials with these anti-idiotypes have been disappointing [45]. In contrast, Abagovomab, a murine IgG1 anti-idiotypic mAb that mimics CA125, has shown more promise. In Phase I/II clinical trials 68% of patients developed anti-CA125 antibody responses and 50% of patients demonstrated an ADCC response against CA125 positive tumor cells [46–49]. The side effects were mild and this antibody is currently in Phase II/III clinical trials (MIMOSA/AGO-Ovar-10 trial).

It has been more difficult to show anti-idiotypic antibodies stimulating cellular immunity, although a recent study has shown that 1E10 anti-idiotypic mAb, which recognizes NeuGc-containing gangliosides, induces therapeutic effects in a primary breast carcinoma and a melanoma model. The therapeutic effect was associated with the increment of T cells infiltrating metastases, the reduction of new blood vessel formation and the increase of apoptotic tumor cells in lung nodules. Interestingly, active immunization does not induce measurable antibodies to the 1E10 mAb [50]. The T-cell responses elicited by anti-idiotypic mAbs are believed to be due to the efficient presentation of T-cell mimotopes contained within the complementarity-determining regions (CDRs) of mAbs to APCs *in vivo*. A human monoclonal IgG1 anti-idiotypic antibody, 105AD7, which expressed a T-cell mimotope of CD55 antigen within its CDR, stimulated helper and cytotoxic T-cell responses in over 300 cancer patients with no associated toxicity [51–54]. Two of the osteosarcoma patients were cured of their disease and are alive and well 10 years

posttreatment. However, in a double-blind randomized trial of 105AD7 in colorectal cancer there was no overall survival advantage, suggesting that the T-cell responses were not of sufficient frequency/avidity to clear bulky tumors. One of the complications of T-cell mimotopes or heteroclitic epitopes is that although they stimulate high-avidity T-cell responses against the mimicking epitope, the avidity of the response against the native antigen is 100-fold weaker. To overcome this problem several groups have replaced CDR-H3 with native helper and B-cell epitopes to stimulate immune responses [55–57]. Zaghouani *et al.*, also attempted to replace CDRH3 with class I restricted CTL epitopes. Although, they showed that CTLs could recognize cells transfected with Ig encompassing an MHC class I CTL epitope from the nucleoprotein of influenza virus (NP-Ig) showing that the epitope was presented, the purified Ig was unable to induce CTLs [58,59]. Recent studies with this mouse IgG2b expressing the NP CTL epitope (NP-Ig) have shown that it is possible to stimulate CTL responses if coadministered with the Toll-like receptor agonist dsRNA which upregulates FcγRIV and downregulates FcγRIIb [60]. In contrast, it has been shown that immunizing with a DNA vaccine incorporating CTL epitopes within a human IgG1 or mouse IgG2a framework, termed ImmunoBody® (Scancell holdings plc, Nottingham, UK), without any additional adjuvants stimulates high-frequency responses to a wide range of epitopes [61].

ImmunoBody®

ImmunoBody vaccine technology involves the replacement of CDR regions within the framework of an engineered human IgG1 antibody with specific CTL and T-helper cell epitopes (Figure 1).

Antibodies are ideal DNA vectors for stimulating immune responses. These responses are 100–1000-fold more effective than protein, peptide or whole antigen DNA immunization [61].

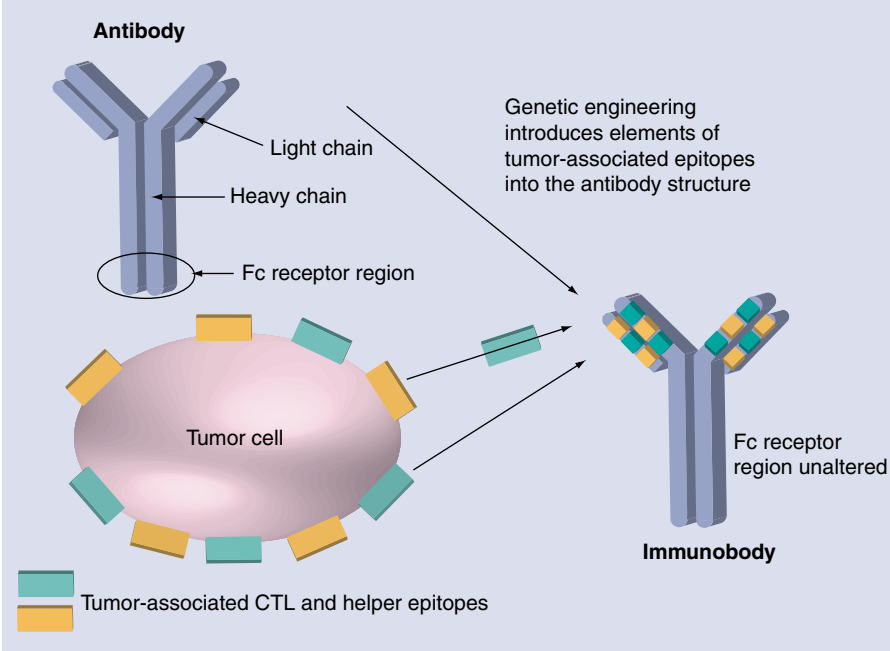

Figure 1. ImmunoBody® technology.

The difference in the frequency of responses generated following injection with the DNA compared with those following administration of the protein equivalent suggests that the direct transfection of skin APCs plays a role in the generation of these immune responses [62]. In the case of an ImmunoBody product, some of the vaccine antigen expressed following uptake of the DNA vector is secreted as an engineered human IgG1 antibody molecule containing the $CD8^+$ and $CD4^+$ T-cell epitopes. This fusion protein would then be able to target (CD64) the FcγR present on APCs via the Fc region, resulting in uptake and cross-presentation of the epitopes to $CD8^+$ and $CD4^+$ T cells. Results have also shown that the FcγR is important in generating high-avidity responses following DNA vaccination, as immunization of FcγR knockout mice results in lower avidity responses [62]. These results suggest that ImmunoBody DNA is presented both directly by transfection of APCs and indirectly by uptake of antibody protein via the high-affinity receptor CD64 resulting in high-avidity and frequency T-cell responses.

A schematic diagram of the proposed mode of action for ImmunoBody vaccines, including both direct- and cross-presentation is provided in Figure 2.

Injection of plasmid DNA encoding the major CTL rejection epitope from B16 melanoma within the CDR of a human IgG1 antibody induces high avidity $CD8^+$ responses that result in inhibition of B16 tumor growth in C57Bl mice. This same epitope is presented by HLA-A*0201 and is a rejection antigen in melanoma patients. Incorporation of two gp100 tumor-specific CD4 epitopes alongside the $CD8^+$ epitope stimulates high-avidity $CD4^+$ responses in HLA-DR4 transgenic mice. These epitopes can also be presented by HLA-DR7, DR53 and DQ6, which encompasses the majority of the Caucasian population. The T-cell epitopes were encoded in CDRH1 and CDRH3 to provide linked help for the CTL epitope. The presence of CD4 help led to induction of significantly higher frequency $CD8^+$ responses that were more functionally active, secreting IFN-γ, TNF-α and IL-2 and that developed into long-term memory. The CD4 epitopes were also encoded within CDRL1 and CDRL3 as the light chain to increase helper responses. Preclinical evaluation of the vaccine demonstrated stronger antitumor immunity than with the CTL epitope alone. If the DNA is administered by gene gun the animals showed widespread vitiligo. However, if the DNA is administered by intramuscular immunization/electroporation the only toxicity was inflammation at the injection site and no vitiligo. The DNA persisted at the injection site for 90 days and in the lymph nodes for 7 days and induced strong $CD8^+$ responses. Established tumors were significantly inhibited. In combination with depletion of Tregs, which enhance the frequency and

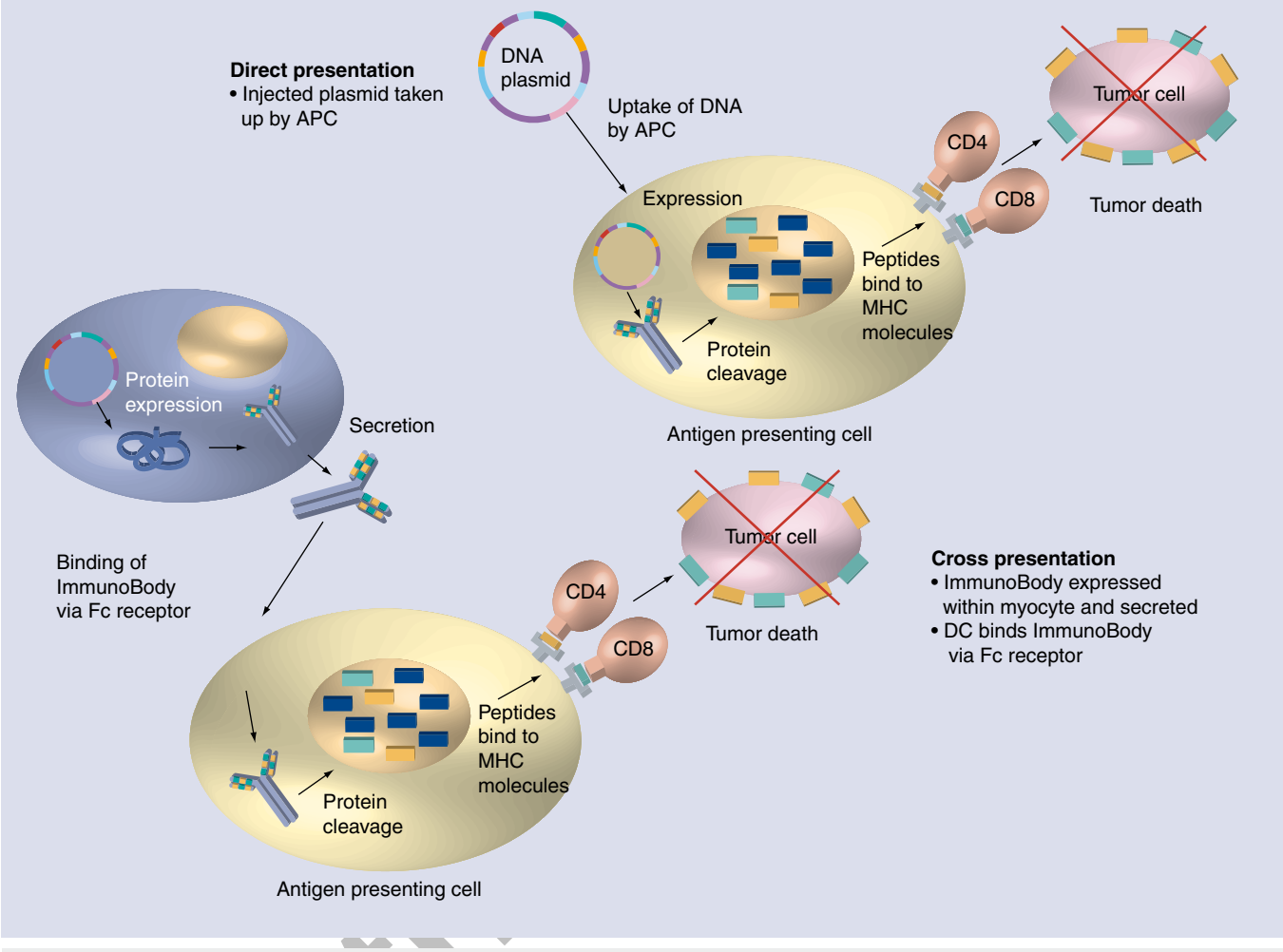

Figure 2. Proposed mechanism of action of ImmunoBody technology.

the avidity of $CD8^+$ but not $CD4^+$ responses, large bulky tumors were completely rejected and 20% of animals where cured. This approach has now been translated into the clinic. SCIB1 (Scancell Holdings plc, Nottingham, UK), is a therapeutic DNA vaccine that is being developed for the treatment of melanoma.

A schematic diagram of the structure of the resulting engineered antibody that would be expressed by SCIB1 is shown in Figure 3.

Various strategies have thus been explored to give better delivery of DNA plasmids, including the use of a gene gun to deliver DNA in a particulate form [63] and electroporation [64]. Electroporation increases transfection efficiency, and therefore antigen expression, by generating controlled electrical pulses to create temporary pores in cell membranes and enable dramatically increased cellular uptake of DNA [65]. SCIB1 has been combined with electroporation-mediated intramuscular delivery using the TriGrid™ deliver system–intramuscular (TDS-IM) electroporation device manufactured and supplied by Ichor Medical Systems Inc (San Diego, CA, USA). Preclinical studies showed that SCIB1 plus electroporation was not toxic but could inhibit the growth of established tumors and prolong survival [62]. The Phase I/II trial is an open label nonrandomized study to determine the safety and tolerability of three dose levels of 0.2, 2 and 4 mg of SCIB1 administered using electroporation with the TDS-IM device and to assess immune effects and antitumor activity in patients with melanoma.

Targeting receptors other than FcγR on APCs

An alternative approach to replacing CDR regions with T-cell epitopes and using the Fc region to target APCs is to replace the Fc region with antigen and use the variable regions to target specific antigens.

Vaccibodies

Vaccibody proteins are bivalent homodimers, each chain consisting of a scFv targeting unit specific for an antigen expressed by APCs and a hinge and CH3 dimerization region fused to antigen. mAb targeting of B-cell idiotype to MHC class II using a vaccibody approach induced T and B cell responses that protected mice against challenge with tumors [66]. Vaccibody proteins targeting human TLR-2 receptor or CD14 were 100–10,000-fold more potent at stimulating CD4 responses than nontargeted proteins [67].

DEC-205 receptor

DEC-205 is a receptor that facilitates enhanced antigen uptake and presentation in APCs. Hawiger *et al.* observed that when

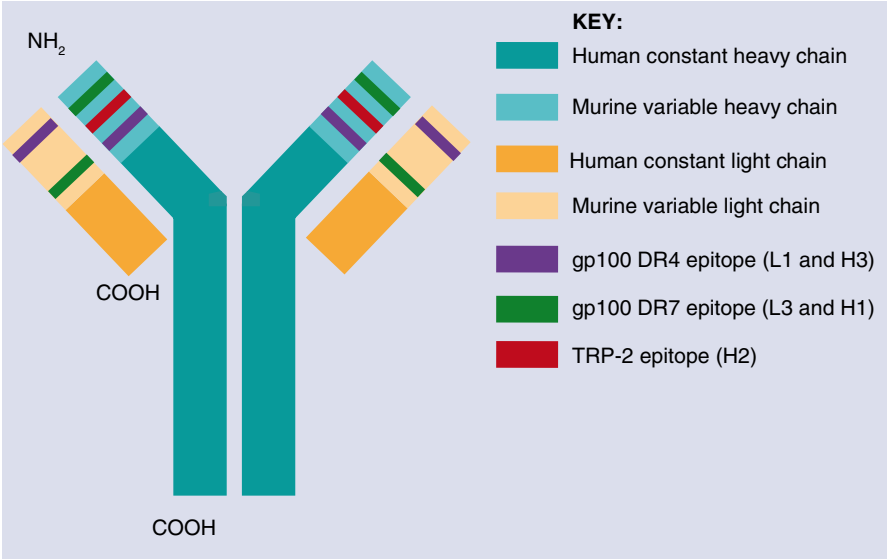

Figure 3. Schematic diagram of the engineered antibody structure encoded by SCIB1.

antigens were targeted to DEC-205 via mAbs, an initial burst of T-cell proliferation was induced, but this was not sustained [68]. Furthermore, a normal T-effector cytokine profile was not induced and the T cells became anergic to antigenic restimulation. In contrast, when anti-DEC-205 targeting was combined with an agonistic anti-CD40 antibody [68], or in conjunction with CpG oligonucleotides, the growth of established melanomas in a murine model was inhibited [69]. More recently, a fusion of xenogeneic survivin to anti-DEC-205 mAb was able to induce $CD4^+$ but not $CD8^+$ responses to this antigen. The CD4 response recognized both human and mouse survivin, thereby, breaking tolerance to this self antigen. However, the avidity of the response to the mouse antigen was 10–100-fold lower than the response to the human protein and these T cells failed to control growth of the A20 lymphoma, which expresses mouse survivin [70]. The same group have also targeted Langerin on DC with mAb/antigen fusion proteins and in a foreign OVA model; preliminary results show similar responses to targeting DEC-205 [71].

In contrast to protein, targeting DEC-205 with scFv/antigen DNA vaccines has been shown to elicit T-cell responses without the need for immune adjuvants [72,73]. Recent studies have shown that when priming with anti-DEC-205/antigen DNA and boosting with adenovirus encoding the same antigen, there was a significantly improved CD8 response after the addition of Toll-like receptor ligands, CpG and poly I:C to the DNA vaccine [74].

More recently a fully human anti-DEC-205 mAb, CDX-1401 (Celldex Therapeutics, Inc.), genetically linked to NY-ESO-1 and in combination with resiquimod, which is an activator of TLR-7 and -8 has completed a Phase I/II clinical trial [201]. Six patients maintained stable disease and were eligible for multiple cycles of the treatment regimen, including four patients who have received three or more cycles (6 weeks of treatment followed by a 6-week rest), with stable disease of up to 11.5 months. The treatment was well tolerated and there were no dose-limiting toxicities. Strong anti-NY-ESO-1 immunity was induced with the majority of the patients developing anti-NY-ESO-1 antibody responses and 39% of the patients having increases in NY-ESO-1-specific T-cell responses, including both $CD4^+$ and $CD8^+$ responses.

Mannose receptor

Several studies have demonstrated that targeting antigens to the mannose receptor (MR) can also improve the efficiency of immunization protocols. A humanized antibody directed against human MR (B11) was generated and genetically fused to either human chorionic gonadotrophin β (hCGβ) or pmel17, a splice variant of the melanocyte-specific protein gp100. In an autologous *in vitro* culture system, hcGβ or pmel17 loaded APC were able to stimulate CD4 proliferative responses, as well as directing the development of CD8 cells [75]. More recently, the use of anti-MR-conjugated antigen has been reported to target antigen to both the exogenous and cross-presentation pathways of antigen presentation in human MR transgenic mice *in vivo* [76]. Furthermore, when co-immunized with CpG-containing oligonucleotides to promote APC maturation, this vaccination strategy was able to induce protective immunity against challenge with a tumor cell line expressing the cognate antigen.

Table 1. Immunomodulatory monoclonal antibodies.

Monoclonal antibody	Antigen	Mechanism of action	Stage	Ref.
Tegeneron (TGN1412)	CD28	Agonist	Failed in Phase I owing to catastrophic cytokine storm	[79]
Tremelimumab (CP-675,206)	CTLA-4	Blocking	Phase III	[101]
Ipilimumab (MDX-010)	CTLA-4	Blocking	Pending approval	[103]
MDX-1106	PD-1	Blocking	Phase II	[125]
CT-011	PD-1	Blocking	Phase I	[126]
Dacetuzumab	CD40	Agonist	Phase I	[131]
CP-870893	CD40	Agonist	Phase I/II	[130]
Anti-OX40	OX40	Agonist	Phase I	[128,129]
BMS 663513	4–1BB	agonist	Phase I	[142]

Immune regulatory mAbs

Although vaccination can lead to the induction of T-cell responses, a more direct approach is to use mAbs to directly activate antitumor T-cell responses. Studies in a variety of systems have shown that engagement of the TCR is not sufficient to activate T cells to full effector function. Further signals by a wide variety of costimulatory molecules are required to amplify and/or modify the TCR signal. Failure to receive this signal can result in anergy or death. Several mAbs have been developed that can bind coreceptors, either inducing (agonist), or inhibiting (blocking) their signaling. This can result in enhanced or reduced immune activation and can result in strong antitumor responses but also in profound toxicity or autoimmunity. Table 1 lists both blocking and agonist mAbs targeting T-cell costimulatory molecules, which are under clinical development. The following sections will discuss the mechanism of action of these mAbs and how effective they have been in the clinic.

mAbs targeting CD28/CTLA-4

The most widely studied costimulatory signal is that provided by B7.1/B7.2 (CD80/86) on APCs to CD28 on activated T cells. Failure to receive this signal can result in anergy or death. Several monoclonal mAbs have been developed that can bind coreceptors, either inducing (agonist), or inhibiting (antagonist) their signaling. This can result in enhanced or reduced immune activation. In the context of tumors, the goal is to enhance the antitumor immune response and/or to relieve the immunouppression/regulation induced within the tumor microenvironment.

Agonist anti-CD28 mAbs – TGN1412

TGN1412 is a humanized superagonist monoclonal antibody of the IgG4κ subclass that stimulates and expands T cells independently of the ligation of the T-cell receptor [77]. In preclinical models TGN1412 (or its anti-mouse counterpart) activated Th2 and Treg cells and resulted in transient lymphocytosis with no detectable toxic or proinflammatory effects [77,78]. In contrast, six healthy volunteers who received TGN1412 at 0.1 mg/kg experienced systemic inflammatory response characterized by rapid induction of proinflammatory cytokines (TNF-α, IFN-γ, IL-2, IL-6 and IL-10) within 1 h that were accompanied by headache, myalgias, nausea, diarrhea, erythema, vasodilation and hypotension. This is a classic cytokine storm response; however, within 12–16 h this had progressed to pulmonary infiltrates, lung injury, renal failure and disseminated intravascular coagulation. At 24 h, all patients had severe lymphopenia, monocytopenia and thrombocytopenia. Four patients responded to intensive cardioplumonary support including dialysis, high-dose methylprednisolone and anti-IL-2-receptor-antagonist antibody. In two patients, cardiovascular shock and acute respiratory syndrome developed and they required intensive organ support for 8–16 days [79]. Fortunately, all six patients survived but use of the drug was discontinued. This was a salutary lesson for people developing mAbs and in particular for targeting molecules that are essential for lymphocyte homeostasis. Following the unexpected toxicity the regulatory authorities have now revised their guidelines for the use of mAbs (MABEL), which required development of a biologically relevant animal model prior to clinical trials and treatment of one patient at a time.

Anti-CTLA-4 mAbs

CTLA-4 is an inhibitory molecule expressed within vesicles in T cells [80]. It is relocated, as a late event during activation, to the cell membrane where it incorporates into the immunological synapse [81] and inhibits TCR signaling [82]. Its role in controlling T-cell responses was exemplified in CTLA-4 knockout mice that die of polyclonal, CD4-dependent lymphoproliferation, which results in fatal tissue destruction of multiple organs within 3–4 weeks of birth [83–85]. CTLA-4 has a 100-fold higher affinity for CD28 than B7.1/B7.2 and thus acts as a competitive inhibitor, effectively blocking T-cell activation [86]. CTLA-4 is also expressed by natural Tregs where it may further block T-cell proliferation [87–89]. Indeed, recent studies have suggested that CTLA-4 may also induce T-cell inhibition by 'back signaling' via B7 into APCs to upregulate indoleamine 2,3-dioxygenase (IDO) [90]. This enzyme is responsible for degradation of tryptophan, which is essential for T-cell proliferation. More recent studies have shown that blocking CTLA-4 promotes Th17 differentiation *in vitro* and *in vivo* and increases the severity of experimental autoimmune myocarditis [91].

Early experiments in mouse models showed that mAbs that blocked CTLA-4 resulted in regression of immunogenic tumors [92–94]. However, less immunogenic tumors also required Treg depletion [95] or active vaccination [96–97]. Potent T-cell responses were generated in all models and were responsible for the antitumor responses. CTLA-4 blockade resulted in increases in the T effector:Treg ratio of both $CD4^+$ and $CD8^+$ cells but the responses were more pronounced in the $CD4^+$ cells [99]. Anti-CTLA-4 mAbs could induce adoptively transferred, IL-17A-polarized transgenic $CD4^+$ cells to express granzyme and perforin and mediate direct tumor killing [100]. Translation of these studies is already underway with two anti-CTLA-4 mAbs in the clinic; tremelimumab (CP-675,206: Pfizer, New York, NY, USA) and ipilimumab (MDX-010: Bristol Myers Squibb, Princeton, NJ, USA).

Tremelimumab

Tremelimumab is a human anti-CTLA-4 in which early clinical trials in melanoma patients demonstrated acceptable toxicity and similar efficacy of 10 mg/kg monthly and 15 mg/kg quarterly regimes with median survival of 10.3 and 11 months, respectively [101]. The latter regime was tested in comparison with standard decarbazine/temozolomide chemotherapy in previously untreated patients. The trial was stopped, based upon a second interim analysis, which failed to show a survival difference between the two groups. At longer follow up and further analysis it was found that C-reactive protein of <1.5 times the upper normal limit seemed to derive a significant survival benefit from treatment with tremelimumab. On this basis, and in this patient population, a Phase III trial comparing tremelimumab versus decarbazine/temozolomide is being discussed.

Ipilimumab

Clinical trials with the human anti-CTLA-4 mAb, ipilimumab, have resulted in complete remissions in about 15% of metastatic melanoma patients with about 40% of patients showing survival benefit [102]. In a recent Phase III trial, ipilimumab, was shown to prolong survival of unresectable stage III/IV melanoma patients with 23.5% alive and ongoing at 2 years [103]. Patients were randomized to receive ipilimumab plus gp100 peptide vaccine, ipilimumab alone or gp100 alone. Patients received 3 mg/kg of ipilimumab every 3 weeks for four treatments. Patients with stable disease for 3 months duration, after week 12, or a partial or complete response were allowed to receive additional doses every 12 weeks until disease progression. The median overall survival in the ipilimumab plus gp100 group was 10 months as compared with 6.4 months ($p < 0.001$) for the gp100 alone group. The rates of overall survival at 2 years in the ipilimumab plus gp100, ipilimumab alone and gp100 alone were 21.6, 23.5 and 13.7%, respectively. Grade 3 and 4 immune-related adverse events occurred in 10–15% of patients, with the most common being diarrhea, which could be treated with prompt administration of corticosteroids or in severe cases infliximab to neutralise TNF-α and prevent a cytokine storm. There was a strong correlation between toxicity and efficacy, which is desirable as toxicity is only acceptable if there is some benefit. Patients showing no benefit had no toxicity and could be removed from the therapy. It was also possible to mitigate the toxicity with steroids, without affecting the antitumor response. Unlike animal studies, it was difficult to measure T-cell responses, although several early trials did show an increase in the T effector:Treg ratio [104]. Ipilimumab was the first drug in the history of melanoma to be approved on the basis of a demonstrated survival benefit in patients with advanced metastatic melanoma. Clinical trials are also ongoing with ipilimumab in prostate, translational cell carcinoma and non-small-cell lung cancer.

mAbs blocking PD-1 mabs

Programmed death-1 (PD-1; CD279) is an inhibitory coreceptor expressed on antigen-activated T and B cells [105]. In contrast to early lethality in CTLA-4 knockout mice, PD-1 knockout mice, demonstrate late-onset strain and organ-specific autoimmunity [106,107]. These results confirmed a role for PD-1 in regulating T-cell tolerance and autoimmunity. There are two known ligands for PD-1: B7-H1 (PD-L1) the predominant mediator of PD-1-dependent immunouppression and B7-H2/PD-L2. B7-H1 is constitutively expressed on B cells, DCs, macrophages, mast cells and T cells and is further upregulated upon activation. It can also be expressed on nonhematopoietic cells including vascular endothelial cell types, epithelial cells and cells at sites of immune privilege including trophoblasts, retinal pigment cells and neurons in the eye. It can also be upregulated in tumors where its expression is associated with poor outcome [108,109]. B7-H2 has a more restricted expression and is induced on DCs, macrophages, peritoneal B1 cells, memory B cells and cultured bone marrow derived mast cells. B7-H1 is expressed within the thymus and has a role in both positive and negative selection [110,111]. It also has a role in peripheral tolerance where PD-1/B7-H1 interactions can inhibit expansion of naive self-reactive T cells and/or inhibit their differentiation to effector T cells [112–114]. Indeed, recent studies have suggested that in the presence of anti-CD3, TGF-β and B7-H1 can induce $CD4^+FOXP3^+$ Tregs from naive $CD4^+$ cells [115]. PD-1 transduces a negative signal when engaged simultaneously with TCR [116,117]. Phosphorylation of the second tyrosine residue recruits the phosphatases, SHP-2 and SHP-1, leading to dephosphorylation of effector molecules activated by TCR and thus regulating the threshold for T-cell activation and the quantities of cytokines produced. Chronic viral infections in humans and in mice are often characterized by 'exhausted T cells', which lose the ability to produce cytokines, lyse infected cells and to proliferate. PD-1 is a marker of exhausted T cells and *in vivo* blockade of PD-1/B7-H1 interactions in chronically infected mice restores T-cell function and leads to enhanced viral control [118]. Similarly, PD-1 expression is increased on human tumor infiltrating lymphocytes [119,120] and PD-1 blockade enhanced expansion and function of human CTL *in vitro* resulting in antitumor immunity in mice [121–123]. On the basis of these results a fully human mAb, MDX-1106, and a humanized IgG1 mAb CT-011 (Curetech Ltd, Yavne, Israel) recognizing human PD-1 have been produced and are being tested in the clinic.

MDX-1106

A total of 39 patients with advanced metastatic melanoma, colorectal cancer, prostate cancer, non-small-cell cancer or renal cancer received a single intravenous infusion of MDX-1106 in dose-escalating cohorts of 0.3, 1, 3 or 10 mg/kg followed by a 15 patient expansion cohort at 10 mg/kg [124]. Patients were retreated if they had evidence of clinical benefit. No maximum tolerated dose was reached with doses up to 10 mg/kg. There was one serious adverse event, an inflammatory colitis in a patient who received five doses of 1 mg/kg. There was one durable complete response (>2 years) in a colorectal cancer patient and two partial responses in a renal and a melanoma patient. In a follow up Phase II trial 21 patients were treated with single dose of 10 mg/kg [125]. Six patients received repeat doses. There was no MDX-1106-related toxicity and one patient had a partial response after three doses, which has lasted for 5 months.

CT-011

CT-011 has been tested in 17 patients with a variety of hematological malignancies and has shown a complete response in one of five lymphoma patients (patient with a stage III follicular lymphoma) and 1 mixed response in an acute myeloid leukemia patient [126]. No maximum tolerated dose was reached with doses up to 6 mg/kg.

Anti-CD40 mabs

CD40 ligand is a member of the TNF-receptor superfamily and is expressed by APCs and other cells including endothelium cells and platelets. Activation of CD40 by binding to CD40 ligand expressed by helper T cells licenses APCs for CTL activation [127]. Anti-CD40 agonist mAbs can overcome the requirement for

T-cell help and can license APCs to mount robust and effect antitumor CTL responses [128,129]. This has lead to the development of a human (CP-870893; Pfizer) and a humanized (Dacetuzumab; SGN-40; Seattle Genetics, Inc. Bothell, WA, USA) anti-CD40 agonist mabs.

CP-870893

CP-870893 is a fully human selective CD40 antibody that has been administered to 29 melanoma patients as a single intravenous administration. The maximum tolerated dose was 0.2 mg/kg, the dose-limiting toxicity being cytokine release syndrome, with high serum levels of TNF-α and IL-6 [130]. Induction of melanoma-specific T cells was seen in the absence of vaccination with any tumor antigen. A second trial combining CP-870893 with chemotherapy resulted in 21% partial responses and 48% stable disease. While vitiligo was seen in two patients, the most prominent side effect was cytokine release syndrome.

Dacetuzumab

A second anti-CD40 mAb, dacetuzumab, was tested in 17 leukemia patients and the single dose maximum tolerated dose was not reached at 6 mg/kg [131]. Clinical benefit was seen in 33% of patients with one complete response. Sustained increase of $CD4^+$ cells was observed for up to 21 days after treatment. In a second trial, patients with refractory or recurrent non-Hodgkin's lymphoma were treated with 2 mg/kg/week for 4 weeks. Additional patients received an intraperitoneal dose of 8 mg/kg. Grade 3 toxicities of anemia, pleural effusions, conjunctivitis, increased liver function and thrombocytopenia were seen. One patient had a complete response and five patients had a partial response.

Anti-Ox40 mabs

Ox40 is another member of the TNF superfamily and is expressed by activated T-helper and CTLs. Binding of Ox40 by its ligand, (Ox40L), augments T-cell activation and effector function [132]. Ox40 is also expressed by Tregs where ligation of the receptor abrogates their suppressive function. Agonist anti-OX40 mAbs have been shown to induce antitumor immunity in mouse models [133–136]. A murine IgG1 antibody was administered to 30 melanoma patients at escalating doses of 0.1 mg/kg, 0.4 mg/kg and 2 mg/kg for three immunizations. The results of the first two cohorts have been published [137]. The antibody was well tolerated. There was some clinical benefit in 6 of 20 patients, which was accompanied by a 2–3 fold increase in $CD4^+$, $CD8^+$ and NK cells. Future studies with a humanized mAbs are required.

Anti-4–1BB mAbs

Activated, but not resting, $CD4^+$ and $CD8^+$ T cells express 4–1BB. Ligation of this receptor results in costimulatory signals that are more potent in $CD8^+$ than $CD4^+$ T cells [138–140]. Treatment of tumor-bearing mice with an agonist 4–1BB mAb stimulated antitumor immunity that was dependent upon $CD8^+$ cells, IFN-γ and CD40 [141]. The mAb did not stimulate $CD8^+$ cell proliferation but prolonged the survival of the tumor-specific $CD8^+$ T cells. A fully human mAb, BMS-663513 (Bristol Myers Squibb), has been administered to metastatic renal, melanoma and ovarian cancer patients at escalating doses of 0.3, 1, 3, 6, 10 and 15 mg/kg [142]. mAb was given every 3 weeks for 4 injections, with retreatment for stable disease or better. Toxicities were minor and of the 47 melanoma patients, three had a partial response and 6 had stable disease. A randomized Phase II trial at doses of 1, 3, and 10 mg/kg is planned.

Expert commentary

Stimulating T-cell responses to tumor-associated antigens is difficult as the repertoire of T cells recognizing these antigens may be deleted or heavily regulated. One approach that is looking very promising is to target antigens to receptors expressed on activated APCs. Several groups have tried using fusion proteins of mAbs and antigens but they suffer from many problems. The fusion proteins have difficulty folding and are produced in very low amounts. They require immune adjuvants to activate the APCs. The highest binding epitopes within self antigens are frequently recognized by natural Tregs. Many of these problems have been resolved by immunizing with DNA encoding the mAbs genetically engrafted with T-cell epitopes. This allows both direct transfection of the APCs but also indirect cross-presentation by protein produced by transfected cells. The low dose and prolonged expression results in high-avidity potent $CD8^+$ T cells that efficiently recognize and kill tumor cells. Incorporation of epitopes rather than antigens allows the vaccine to focus the immune response to new subdominant epitopes overcoming both regulation and immune evasion. Finally, the DNA encodes CpG motifs that stimulate TLR-9 and is also recognized by cytosolic mediators of stress such as DNA-dependent activator of interferon regulatory factors (DAI), which release proinflammatory cytokines and thus DNA acts as its own adjuvant. This competition between tumor growth and the immune system can persist for years and can continue to restrict tumor growth even after the primary tumor has spread to secondary sites. Indeed, in a number of tumors, T-cell infiltration correlates with favorable prognosis. In these patients it may be possible to intervene and to tip the balance in favor of the immune system, thus eradicating tumors. mAbs targeting coreceptors on T cells are one way of achieving this objective. However, the unexpected toxicity associated with TGN1412 has made it essential to screen in biological relevant animal models. It may be safer to block rather than agonise receptors, as the former are more controllable. However, nonspecific immune stimulation and/or blocking will always be associated with systemic autoimmune responses. The severity of these responses is only acceptable if the mAb is effective. The pronounced impact of ipilimumab on survival in advanced melanoma patients is remarkable but does come at a cost of significant toxicity and, in some cases, the death of patients. Although clinicians are now better at treating the toxic symptoms and deaths are rare there is still considerable morbidity. The anti-PD-1, CD40, Ox40 and 4-1BB mAbs induce less toxicity but so far their clinical responses in Phase I trials have been modest. These mAbs will need to show greater efficacy in Phase II trials to justify their continued development. Combinations of mAb vaccines targeting subdominant epitopes to activated APCs and mAbs targeting costimulatory receptors may provide effective therapy for a broad range of tumors.

Five-year view

In the next five years ipilimumab will be approved for the treatment of stage IV and probably stage III melanoma. Ipilimumab will also be approved for use in other cancers. At least one other anti-costimulatory targeting mAb will be approved, probably, MDX-1106. Following on from the success of ipilimumab, mAbs targeting other T-cell costimulatory antigens will enter the clinic. However, in patients whose tumors have escaped detection by their *in situ* immune responses these nonspecific stimulations will be ineffective. As this accounts for over 70% of patients new ways of stimulating *de novo* immune responses in patients are required. It is our belief that DNA vaccines targeting APCs in combination with electroporation will finally reach clinical efficacy and will be approved for a human indication. Combinations of vaccines and mAbs targeting T-cell costimulatory antigens should have an even more a marked impact on patient prognosis. These successes will yield a decade of effective and novel vaccines being approved for the treatment of cancer.

Financial & competing interests disclosure

Lindy G Durrant is a director and Victoria Brentville an employee of Scancell holding plc than own the ImmunoBody technology. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Antibody-targeted DNA vaccines in combination with electroporation show potent antitumor responses in animal models and are now entering the clinic.
- Monoclonal antibodies (mAbs) that target antigens to antigen-presenting cells stimulate high avidity T cell responses that result in regression of solid tumors.
- mAbs that block inhibitory costimulatory molecules on T cells stimulate effective antitumor immunity.
- Ipilimumab, an anti-CTLA-4 mAb, has been approved for treatment of advanced melanoma.
- mAbs that stimulate costimulatory molecules on T cells can also stimulate effective antitumor immunity.

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